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Pou5f1 contributes to dorsoventral patterning by positive regulation of *vox* and modulation of *fgf8a* expression

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ABSTRACT

Pou5f1/Oct-4 in mice is required for maintenance of embryonic pluripotent cell populations. Zebrafish *pou5f1* maternal–zygotic mutant embryos (*spiel ohne grenzen*; MZ*spg*) lack endoderm and have gastrulation and dorsoventral patterning defects. A contribution of Pou5f1 to the control of *bmp2b*, *bmp4* and *vox* expression has been suggested, however the mechanisms remained unclear and are investigated in detail here. Low-level overexpression of a Pou5f1-VP16 activator fusion protein can rescue dorsalization in MZ*spg* mutants, indicating that Pou5f1 acts as a transcriptional activator during dorsoventral patterning. Overexpression of larger quantities of Pou5f1-VP16 can ventralize wild-type embryos, while overexpression of a Pou5f1-En repressor fusion protein can dorsalize embryos. Lack of Pou5f1 causes a transient upregulation of *fgf8a* expression after mid-blastula transition, providing a mechanism for delayed activation of *bmp2b* in MZ*spg* embryos. Overexpression of the Pou5f1-En repressor induces *fgf8*, suggesting an indirect mechanism of Pou5f1 control of *fgf8a* expression. Transcription of *vox* is strongly activated by Pou5f1-VP16 even when translation of zygotically expressed transcripts is experimentally inhibited by cycloheximide. In contrast, *bmp2b* and *bmp4* are not activated under these conditions. We show that Pou5f1 binds to phylogenetically conserved Oct/Pou5f1 sites in the *vox* promoter, both in vivo (ChIP) and in vitro. Our data reveals a set of direct and indirect interactions of Pou5f1 with the BMP dorsoventral patterning network that serve to fine-tune dorsoventral patterning mechanisms and coordinate patterning with developmental timing.

Introduction

The transcription factor Pou5f1/Oct4 controls pluripotency of mouse embryonic inner cell mass cells (Nichols et al., 1998) and ES cell lines (Boiani and Scholer, 2005), and is an essential factor for somatic cell reprogramming (Takahashi and Yamanaka, 2006). *Pou5f1* gene homologues were identified in all vertebrate phyla starting from Gnathostomes: birds (Lavial et al., 2007), *Xenopus* (*XlPou91*, *XlPou25*, and *XlPou60*) (Hinkley et al., 1992), axolotl (Bachvarova et al., 2004), and zebrafish (Takeda et al., 1994). *Pou5f1* homologues show broad expression during pre-gastrulation and gastrulation stages (Bachvarova et al., 2004; Belting et al., 2001; Burgess et al., 2002; Downs, 2008; Lavial et al., 2007; Lunde et al., 2004; Morrison and Brickman, 2006), suggesting that their function is conserved at least in part during these stages. However, mammalian Pou5f1/Oct4 has evolved additional functions beyond Pou5f1/Pou2 (Niwa et al., 2008).

In mouse, loss of Oct4 in the embryo results in developmental arrest before the blastocyst stage (Nichols et al., 1998), precluding the analysis of Oct4 functions at later stages. Functional loss of Pou5f1 homologues in early development of lower vertebrates produces severe gastrulation and patterning abnormalities and results in embryonic lethality. Zebrafish *pou5f1* maternal–zygotic mutant embryos (*spiel ohne grenzen*; MZ*spg*) lack the endodermal germ layer, have gastrulation defects, and are dorsalized. Pou5f1 appears to have independent input into each of these developmental processes (Lachnit et al., 2008; Lunde et al., 2004; Reim et al., 2004; Reim and Brand, 2006). In *Xenopus*, simultaneous loss-of function for three co-orthologues of Pou5f1 (Xlpou25, Xlpou60 and Xlpou91) (Morrison and Brickman, 2006), or double knockdown of Oct25 and Oct60 (Cao et al., 2006), produces excess of endoderm, neuralization of the ectoderm and gastrulation defects. Although effects of Pou5f1

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loss on germ layer development differ between *Xenopus* and zebrafish, loss-of function phenotypes in both species can be rescued by overexpression of mouse Oct4 (Cao et al., 2006; Morrison and Brickman, 2006; Onichtchouk et al., 2010) arguing for conserved ancient roles of Pou5f1 proteins during gastrulation of vertebrates. A better understanding of interactions of Pou5f1 and its multiple downstream transcriptional targets with signaling pathways involved in patterning the embryo should shed light on mechanisms that coordinate control of pluripotency, cellular differentiation, and pattern formation. Further knowledge gained from studying *pou5f1* in different vertebrate organisms could aid efforts toward controlled ES cell differentiation.

Patterning of the vertebrate body axes relies on several signaling pathways that are active during early embryonic development and interact with each other to establish a genetic network, which specifies cell fates along the dorsoventral extent of the embryo. The best understood pathways in early embryonic patterning include the TGF-β (BMP, Nodal), WNT, and FGF pathways (for a review see Schier and Talbot, 2005).

Specification of dorsoventral cell fates is achieved by a BMP activity gradient along the dorsoventral axis (De Robertis et al., 2000). In zebrafish, as soon as the zygotic genome becomes activated, the expression of *bmp2b* and *bmp7* is initiated throughout the blastoderm. Within 30 min after beginning of sphere stage, *bmp2b* and *bmp7* transcripts become ventrally restricted due to transcriptional repression from the dorsal side, a process controlled by the transcriptional repressor Bozozok (Leung et al., 2003) and the FGF signaling pathway (Furthauer et al., 2004). The robustness of the molecular network generating the dorsoventral BMP activity gradient is ensured by cooperation of several partially redundant interdependent pathways. In zebrafish, dorsal inhibition of BMP signals during gastrulation is achieved by combined action of FGF signaling (Furthauer et al., 2004), and three dorsally expressed secreted molecules: Chordin (Schulte-Merker et al., 1997), Noggin 1 (Furthauer et al., 1999), and Follistatin-like 2 (Dal-Pra et al., 2006).

Experimental embryology demonstrated an essential role for Chordin in inhibiting ventralizing BMPs, thereby promoting the development of dorsal fates (Piccolo et al., 1996). While Chordin is the main player in establishing the proper level of BMP activity along the DV-axis, other factors, such as noggin and follistatin, play redundant roles (Dal-Pra et al., 2006). Anti-dorsalizing morphogenetic protein (ADMP) is a BMP receptor ligand that suppresses dorsal and anterior structures when overexpressed. In contrast to other BMP receptor ligands it is expressed dorsally, and maintained by Nodal signaling and indirect action of the transcription factor Bozozok (Lele et al., 2001). Results in zebrafish suggest that ADMP signaling causes the restriction of anterior and axial fates and cooperates with BMP signaling in establishing proper dorsoventral regionalization (Willot et al., 2002). A role of opposing ADMP and BMP signaling in providing self-regulating capacity of the dorsoventral gradient in the embryo was recently suggested (Ben-Zvi et al., 2008; Reversade and De Robertis, 2005).

WNT canonical signaling plays a dual role with respect to dorsoventral polarity in the zebrafish embryo: maternal WNT signaling induces the organizer before MBT, and zygotic WNT restricts organizer function during blastula and gastrula stages by maintaining the expression of the ventral homeobox transcriptional repressors Vox, Vent and Ved (Gilardelli et al., 2004; Kawahara et al., 2000a, 2000b; Melby et al., 2000; Shimizu et al., 2002). Expression of Vox, Vent and Ved is initiated by the maternally expressed transcription factor Runx2b (Flores et al., 2008) and maintained by WNT signaling at blastula until midgastrula stages (Ramel and Lekven, 2004; Varga et al., 2007). The positive cross-regulatory loop between Bmp pathway and Vox/Vent genes is first established at midgastrulation (Imai et al., 2001; Kawahara et al., 2000a, 2000b; Melby et al., 2000; Ramel and Lekven, 2004), while at earlier stages Vox, Vent and Ved are relatively independent of Bmp signaling.

Zebrafish Pou5f1 has been implicated in dorsoventral patterning based on the dorsalization of MZ*spg* mutants during gastrula stages, as judged from shifts in gene expression domains. It was previously shown to enhance transcription of *bmp2b* and *bmp4* genes upstream of the maternal Alk8 receptor, and to be involved in maintaining *vox* and *vent* expression (Reim and Brand, 2006). However, it remained unknown, whether the interactions between Pou5f1 and the components of dorsoventral patterning pathways are direct. Here, we combine analysis of MZ*spg* mutants by time-resolved transcriptional profiles for the genes involved in the BMP regulatory circuitry, and of overexpression of activator and repressor Pou5f1 fusion constructs. We employ assays with protein synthesis inhibition experiments to identify potentially direct interactions. Shortly after MBT in MZ*spg* embryos, we demonstrate a transient ectopic overactivation of expression of the dorsalizing genes *fgf8a*, *chd* and *noggin1*, and present evidence that *fgf8a* overactivation is caused by indirect action of Pou5f1. We show that Pou5f1 contributes to dorsoventral patterning by direct activation of the *vox* promoter, and that this is a phylogenetically conserved mechanism.

Materials and methods

Fish manipulation and care

Zebrafish embryos and larvae were raised at 28.5 °C in 0.3× Danieau's solution (Shih and Fraser, 1996). WT embryos of AB×TÜB strain crosses and MZ*spg* carrying the *m793* allele of the *spg* mutation were used (Belting et al., 2001). Developmental staging was performed according to Kimmel et al. (1995). Photographs of live embryos in 2% methylcellulose, 0.4% tricaine in 0.3× Danieau's solution, or of fixed embryos in 70–100% glycerol were taken with an Axiocam digital camera using a Zeiss Axiophot 2 or a LeicaMZ10 dissecting microscope.

Whole-mount in-situ hybridization and immunostaining

Whole-mount in situ hybridization was performed to visualize gene expression as described (Belting et al., 2001). The following probes were used: *bmp2b* (Kishimoto et al., 1997), *bmp4* (Martinez-Barbera et al., 1997), *chordin* (Miller-Bertoglio et al., 1997), *fgf8a* (previously called *fgf8*; Furthauer et al., 2004), *vox* and *vent* (Melby et al., 2000), and *ntl* (Schulte-Merker et al., 1994). Anti pSmad1/5/8 antibody against the phosphopeptide (NH2-CNPIS-S [PO3]-V-S [PO3]-COOH) was kindly provided by Dr. Ed Laufer and used in 1/500 dilution for anti-P-Smad immunostaining performed as in Stemple et al. (1996).

Plasmids and mRNA injection

For sense mRNA preparations: *Pou5f1-En* and *Pou5f1-VP16* fusion proteins have been described in Lunde et al. (2004). *caAlk3* (Nikaido et al., 1999), *fgf8a* (Furthauer et al., 2004) *Smad1* (Dick et al., 1999) and membrane-GFP from the RN3-EGFP-F plasmid (Weidinger et al., 2002) were also used. Dr. A. Tomilin kindly provided Mouse expression constructs CS2 + Oct4 and CS2 + mycOct4. To obtain the Vox-Luc reporter construct, 1 kb upstream of vox coding sequence (ZFISH7:13:52347190:52350403) was PCR amplified from zebrafish genomic DNA, using PCR primers with incorporated KpnI/BglII sites, cloned into PCRII-Topo vector, sequenced and subcloned into pGL4.26 (Promega) using KpnI/BglII sites. 3× FLAG-TAG sequence was amplified from the P3XFLAG-CMV-7-vector (Sigma-Aldrich, Catalog# E7408) and added 3′ to *pou5f1* by fusion PCR. The construct was subcloned into PCRII-Topo vector and sequenced. Finally the construct was EcoRI/XhoI cloned into the expression vector pCS2+. Primers used for cloning (5′ to 3′) were

Eco-Pou: GAATTCATGACGGAGAGAGCGCAGAG
FlagFuspou-rev: TCGCTGGTGAGATGACCCAC

FlagFuspou-rev: TCGCTGGTGAGATGACCCAC
Xho-Flag-rev: CTCGAGTTACTTGTCATCGTCATCCT.

Capped sense RNA was synthesized using the mMESSAGE mMACHINE SP6 system (Ambion), dissolved in 0.1 M KCl, and microinjected into one- to two-cell stage embryos. The actual amounts (pg) injected into embryos were calculated using the drop diameter as measured using an object micrometer and ruler in the ocular lens of the microscope. Experiments shown in Figs. 1A and 4B were also controlled for injection amounts by co-injecting 22–100 pg membrane-GFP mRNA (Weidinger et al., 2002), and embryos not expressing GFP were removed.

Cycloheximide experiment

Embryos were injected with mRNA or left non-injected, and were treated with protein inhibitor cycloheximide (CHX, Calbiochem), 15 μg/ml in egg water (Leung et al., 2003), starting from 1.5 hpf until

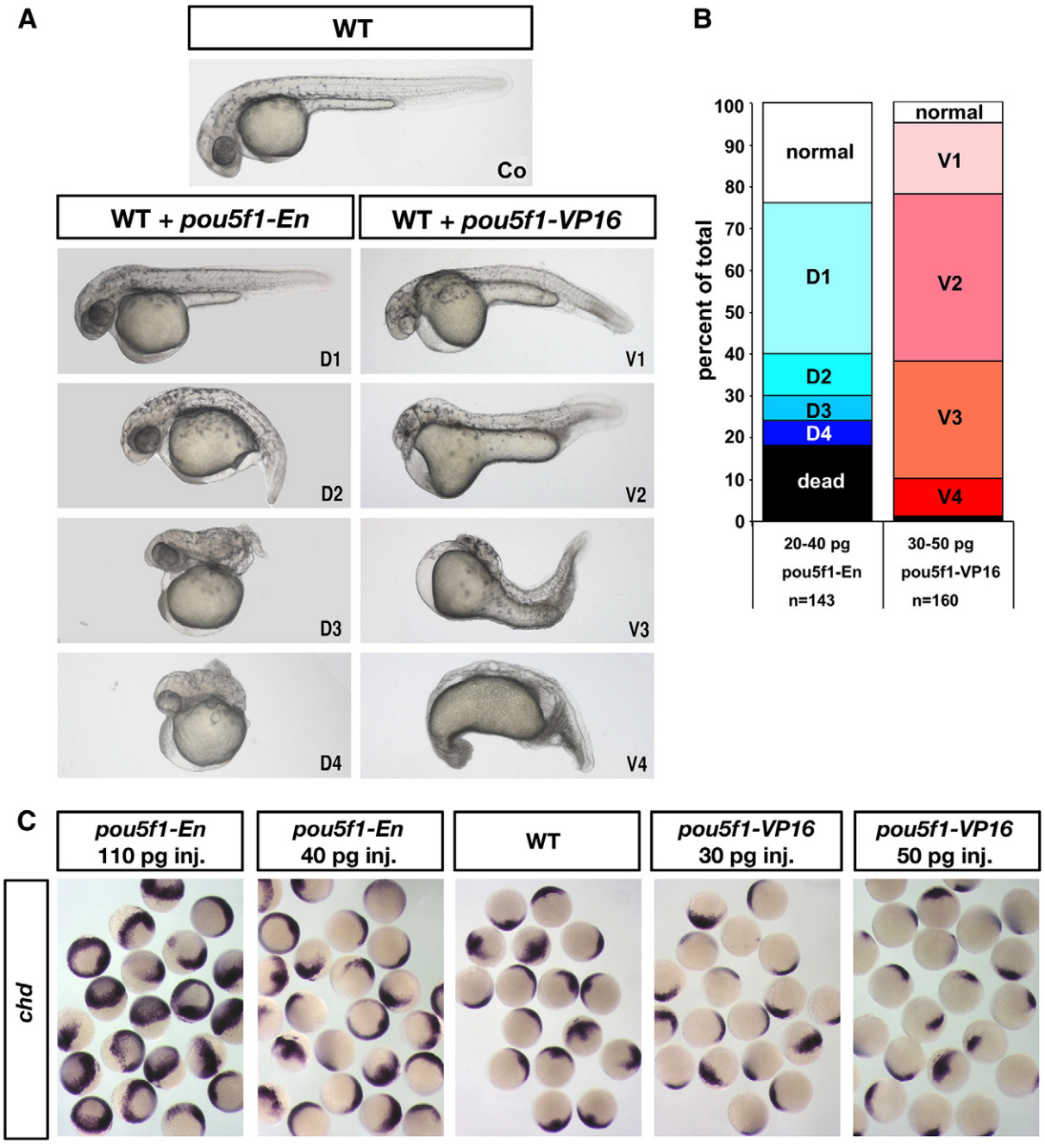

Fig. 1. Overexpression of Pou5f1-VP16 can ventralize wild-type embryos while overexpression of Pou5f1-En can dorsalize. (A) Live photographs of a wild-type (WT) zebrafish embryo at 33–36 h post fertilization (28.5 °C) (top) and of embryos that were injected with *pou5f1-En* mRNA (20 pg; D1–D4) or *pou5f1-VP16* mRNA (40 pg; V1–V4) at the one-cell stage within 30 min of egg laying. As an injection control, membrane-GFP mRNA (30 pg) was co-injected, and embryos with low GFP expression at early shield stage were removed. Lateral views, anterior to left, dorsal at top. Dorsoventral index modified from Kishimoto et al. (1997). (B) Dorsoventral index of WT zebrafish embryos scored at 33–36 hpf. The graph shows percent of phenotypes (V1–V4, D1–D4 as in (A), dead and normal) from total number of injected embryos. (C) Whole-mount in situ hybridization of pooled WT embryos injected with *pou5f1-VP16* mRNA (30 or 50 pg) or *pou5f1-En* mRNA (40 or 110 pg) at the one-cell stage, and of non-injected WT control embryos, showing expression of *chordin* at 70% epiboly.

the embryos were frozen for RNA isolation. In presence of CHX, direct Pou5f1 targets are transcribed, but these mRNAs are not translated, thereby avoiding indirect downstream regulatory effects. CHX was added at the 64-cell stage to allow for translation of injected *pou5f1* mRNA, but to block translation of the earliest zygotic transcripts. Loss of *ntl* expression in CHX embryos was used as control for efficient inhibition of translation.

Quantitative RT-PCR

60–100 embryos per sample were snap-frozen in liquid nitrogen, and total RNA was isolated using the RNA Easy kit (Qiagen). cDNA was synthesized using Superscript III kit (Invitrogen). cDNA was amplified using gene-specific primers and Absolute SYBR QPCR green mix (Thermo Scientific) according to the manufacturer's instructions on an Eppendorf Realplex light cycler. Results were calculated using ddCT method and zebrafish *ef1a* (cDNA measurements) or ribosomal protein *rpl5b* (CHIP) as a normalization control. For *chordin* and *admp*, the Roche UPL system was used; UPL probe number is indicated in the primer name.

Zebrafish primers	Sequence 5′–3′
ef1a_f	CCTGGGAGTGAAACGCTGATC
ef1a_r	CCGATCTTCTTGATGTATGCGCTG
vox-Q-f1	GCTTGCTCAGAGCTTTCATG
Vox-Q-r2	CATAACCCGACGAATAACCG
rpl5b_f (CHIP)	GGGGATGAGTTCAATGTGGAG
rpl5b_r (CHIP)	CGAACACCTTATTGCCAGTAG
ChIP_Vox_f5	GATGCAGACAGCTGGATGAG
ChIP_Vox_r4	ACGCCTGATCGATTCTCAAT
ef1a_UPL_73f	CCTCTTTCTGTTACCTGGCAAA
ef1a_UPL_73r	CTTTTCCTTTCCCATGATTGA
Admp_UPL_2f	GCTGCATCTCTGGAGGCTAC
Admp_UPL_2r	ACTGGGAAGCGGCTGACT
Chordin_UPL_50f	AGCAAACATCACTGCAGACG
Chordin_UPL_50r	GGGTTTGACCTTCAGTCTGG

Luciferase assays

Zebrafish embryos were microinjected with 15 pg/embryo of Vox-luc, 5 pg/embryo of pGL4.75[hRluc/CMV] Vector (Promega) and mRNAs (10–100 pg/embryo) as indicated. At 6 hpf, 40–60 embryos per assay were homogenized and snap-frozen in the lysis buffer. Samples were further processed according to the manufacturer's instructions (Dual luciferase reporter assay system, Promega). Luciferase and renilla activity were measured on a Bechthold luminometer. Luciferase vector with minimal promoter (pGL4.26 [luc2/minP/Hygro] Vector, Promega) was included as a negative control and showed no specific activation. Each experiment was done in biological and technical triplicates. Results were calculated as luciferase to renilla ratio and normalized to control.

Gel retardation assays

P1–P4 double-stranded oligos were synthesized, with GGG overhangs added at 5′ ends for labeling (Fig. S1). The genomic regions used for oligo design (Zv7 *Danio rerio* genome assembly) at chr13: P1: 52,348,097–52,348,114; P2: 52,348,024–52,348,042; P3: 52,347,724–52,347,741; P4: 2,347,648–52,347,666. zPou5f1, Zebrafish (z)Pou5f1, mouse Oct4 and mycOct4 proteins were synthesized with TNT® SP6 Quick Coupled Transcription/Translation System (Promega) using SP6 expression plasmids. One fourth of the translation reaction was biotinylated using Transcend™ tRNA (Promega), and the products were visualized on Western blot, to ensure that equal quantities of proteins were synthesized. Oligos were labeled with Klenow labeling reaction with dCTP, alpha [32P]- (Hartmann Analytics). Gel retardation assays were performed according to Ausubel et al. (2009). Specific complexes with zPou5f1, Oct4 and mycOct4 were detected when using labeled P1, but not P2–P4 probes (Fig. 6B and data not shown). The formation of labeled Oct4/P1 and zPou5f1/P1 complexes was effectively prevented with as little as 10-fold excess of unlabelled P1, but not P2–P4 oligos (Fig. S2 and data not shown). Mouse Anti-myc antibody (Invitrogen R951-25) was added to the reaction to show specificity of mycOct4/P1 complex in the supershift (Fig. S2).

Chromatin immunoprecipitation (CHIP)

CHIP was performed for shield stage embryos as described (Wardle et al., 2006), with the following modifications: More than 1000 embryos were injected with 22 ng/µl Pou5f1-FLAG RNA or left noninjected. The number of isolated nuclei after cell lysis was determined following Sytox staining in a Neubauer counting chamber. The used amount of chromatin was dependent on nuclei number and equivalent to 4.5 10^6 nuclei for both WT and injected embryos. 9 µg anti-Flag antibody (Sigma Aldrich, F1804) was coupled to 200 µl Invitrogen Dynabeads Protein G (Cat: 100.04D) for each set of WT and Pou5f1-Flag injected embryos. Chromatin was sonicated using Covaris S2 sonicator, DC 20%, Intensity 5, Cycles of Burst 200, and Time = 40 30 s (20 min) to an average size of 150 basepairs. The relative amount of Vox upstream region in immunoprecipitated chromatin and input control were measured using QPCR with SYBR green mix (Thermo Scientific) according to the manufacturer's instructions on Eppendorf Realplex light cycler. DNA abundance was calculated using the second derivative method (Luu-The et al., 2005). DNA abundance was normalized to the input control for injected and non-injected samples to calculate fold enrichment.

Primers	Sequence 5′–3′
rpl5b_f	GGGGATGAGTTCAATGTGGAG
rpl5b_r	CGAACACCTTATTGCCAGTAG
ChIP_Vox_f5	GATGCAGACAGCTGGATGAG
ChIP_Vox_r4	ACGCCTGATCGATTCTCAAT

Homology searches and phylogenetic alignment

Homology searches were performed using the BLAT alignment tool (Kent, 2002) in the UCSC genomic server. Sequences of 5′ upstream *vox* promoter regions of teleost fish species were aligned using the ClustalW2 program in the EBI server www.ebi.ac.uk and visualized using Jalview (Waterhouse et al., 2009) (Fig. 6A). The following genome assemblies and sequences were used:

tetNig1_ chrUn_random:138310610–138310795 strand = −
fr2_dna range = chrUn:116511230–116511401 strand = +
gasAcu1_dna range = chrVI:2022670–2022858 strand = +
oryLat2_dna range = chr15:1377417–1377593 strand = +
danRer6_dna range = chr13:50033974–50034149 strand = −.

Microarray-based transcriptome analysis

The existing microarray data from Onichtchouk et al. (2010) were used in this study. The primary microarray data from the Agilent developmental curve microarrays were stored under accession series number GSE 17667 in GEO (http://www.ncbi.nlm.nih.gov/geo/).

Results

Pou5f1 directly promotes expression of ventral-specifying genes

Zebrafish *pou5f1* is expressed from the maternal genome during oogenesis and blastula stages (Takeda et al., 1994), and lack of Pou5f1 in MZ*spg* causes dorsalization of the embryos (Reim and Brand, 2006).

To distinguish whether Pou5f1 contributes in a positive or negative manner to the control of expression of dorsoventral patterning genes, we injected mRNA encoding C-terminal fusion proteins of Pou5f1 with the transcriptional activator domain VP16 (*pou5f1-VP16*) or the repressor domain of Engrailed2 (*pou5f1-En*) (Lunde et al., 2004) into wild-type embryos at the one-cell stage, and allowed them to mature to 1.5 days post fertilization (dpf). *pou5f1-VP16* overexpression induced various degrees of ventralization in live embryos, while overexpression of *pou5f1-En* resulted in dorsalization (Fig. 1A and B). To investigate effects on gastrula patterning, we performed similar experiments by injecting increasing mRNA amounts and examining the expression of *chordin* at 70% epiboly. We observed increasing expansion of the *chd* domain to lateral and ventral regions of the embryo with increasing *pou5f1-En* concentrations, and progressive restriction of the *chd* domain to dorsal regions resulting from increasing *pou5f1-VP16* overexpression (Fig. 1C). Given that loss of function of *pou5f1* in MZ*spg* causes dorsalization, the identification of dominant active Pou5f1-VP16 as a ventralizer, and of the dominant-negative Pou5f1-En as dorsalizer (phenocopying the MZ*spg* phenotype) suggests that Pou5f1 in the embryo predominantly acts as an activator of gene expression promoting ventral fates.

Pou5f1 indirectly activates bmp genes and represses fgf8a after MBT

bmp2b and *bmp7* transcripts are expressed first at the sphere stage (4 hours post fertilization, hpf), shortly after the onset of expression of the zygotic genome, whereas *bmp4* transcripts first become detectable one hour later. At sphere stage, expression of *bmp2b* and *bmp7* is detected throughout the blastoderm in wild-type (WT) embryos. Initiation of expression of *bmp* genes is delayed or reduced in MZ*spg* mutants (Reim and Brand, 2006). To test whether Pou5f1 activates early *bmp* gene expression, we injected *pou5f1-VP16* into WT embryos, and evaluated *bmp2b* transcript abundance in injected and non-injected embryos. While Pou5f1-VP16 did not over-activate *bmp2b* at stages up to 50% epiboly, Pou5f1-En completely repressed *bmp2b* in the animal blastomeres (Fig. 2A). We did not observe changes in early *bmp7* expression or premature induction of *bmp4* at 4 hpf in *pou5f1-VP16* injected embryos (data not shown). Pou5f1-VP16 repressed the mesodermal marker *no tail*, as we saw gaps in the circular expression of *no tail* in Pou5f1-VP16 injected embryos at 50% epiboly (23 embryos, n = 27; Fig. 2B). *pou5f1-En* injection produced the opposite effect, resulting in thickening of the marginal *no tail* expression domain, causing it to extend animal-wards (Fig. 2B). *no tail* expression in the dorsal domain depends on Nodal and FGF pathway signaling in zebrafish, and inhibition of *no tail* expression similar to what is shown (Fig. 2B) can be seen in Nodal pathway mutants (Feldman et al., 1998) or achieved by overexpression of dominant-negative FGF receptor (Griffin et al., 1995). Differential effects of Pou5f1-VP16 and Pou5f1-En on *no tail* expression raise the possibility that Pou5f1-VP16 and Pou5f1-En indirectly interfere with Nodal or FGF signaling (Griffin et al., 1995). Indeed, overexpression of Pou5f1-VP16 repressed both *ndr1* (Nodal ligand Squint, (Feldman et al., 1998); Fig. 2C, 18/28 embryos) and *fgf8a* (Fig. 2D, 22/26 embryos). In contrast, injection of Pou5f1-Engrailed resulted in the local thickening of the marginal rings of expression of *ndr1* and *fgf8a*.

Active FGF signaling is necessary for transcriptional repression of *bmp* on the dorsal side of sphere stage embryos, and is elicited by FGF ligands Fgf8, Fgf3 and Fgf24 which function redundantly (Furthauer et al., 2004). Overexpression of any of these ligands represses *bmp2b* in the non-marginal blastomeres, but not in the margin and yolk-syncytial layer (Furthauer et al., 2004), similar to the effects observed following Pou5f1-En overexpression (Fig. 2A). To address the possibility, that *bmp2b* repression results from inappropriate activation of FGF signaling in MZ*spg* embryos, we compared the expression of *fgf8a* in WT and MZ*spg* embryos at sphere stage. While transcripts for *fgf8a* in WT embryos first appear dorsally at sphere stage (Furthauer et al., 2004), in MZ*spg* embryos *fgf8a* was activated throughout the whole margin (Fig. 2E and F). Upon overexpression of the Pou5f1-En dominant-repressive construct in WT embryos, we observed the spreading of *fgf8a* expression domain to the whole margin at dome-30% epiboly stage (Fig. 2G; 7 out of 8 embryos), similar to the distribution of *fgf8a* transcripts in MZ*spg* embryos. Experiments with dominant-activator and dominant-repressor constructs suggest that Pou5f1-VP16 may activate, and Pou5f1-En may repress a hypothetical repressor of *fgf8a*.

Since FGF signaling represses transcription of *bmp* genes (Furthauer et al., 2004), ectopic activation of *fgf8a* expression in MZ*spg* embryos may explain the previously reported (Reim and Brand, 2006) suppression of *bmp2b* at sphere stage. However it was also demonstrated previously that *MZspg* mutants exhibit no response to overexpression in the neural plate (Reim and Brand, 2002). To determine whether transient increase in the *fgf8a* expression may mediate reduction of *bmp2b* in MZ*spg* embryos, we compared *bmp2b* expression levels at dome-30% epiboly stage in WT and *MZspg* controls and embryos microinjected with *fgf8a* mRNA. In both *fgf8a* injected WT and MZ*spg* embryos the levels of *bmp2b* message were dramatically reduced (Fig. 2H, I). In *MZspg* mutants, Fgf8a-overexpression further decreased expression of *bmp2b* below the detection levels (Fig. 2I; 18 out of 18 embryos) indicating that the FGF signaling pathway is active in the MZ*spg* mutant embryos. In contrast to *bmp2b*, little or no change in *vox* expression levels was observed upon FGF8A overexpression (Fig. 2J). Finally, we analyzed whether the expression of *bmp2b* in sphere stage MZ*spg* embryos could be rescued by inhibiting FGF signaling. WT and MZ*spg* embryos were treated with SU5402, or for control with DMSO, and *bmp2b* expression was compared at sphere stage. While inhibition of FGF signaling caused a dorsal spreading of *bmp2b* in WT (Fig. 2K), as reported by Furthauer et al. (2004), we found that in MZ*spg* embryos it caused full rescue of *bmp2b* expression (Fig. 2L).

Taken together, our data indicate that one of the functions of Pou5f1 is to ensure proper temporal control over activation of FGF signaling at or shortly after MBT. In MZ*spg* embryos, this control is absent and premature activation of FGF signaling may contribute to the dorsalized phenotype, repressing *bmp2b* during the early stages. Since *fgf8a* and the FGF target *no tail* are repressed by the dominant-active construct Pou5f1-VP16 (Fig. 2B, D), and since the dominant-repressor construct Pou5f1-En activates *fgf8a* (Fig. 2D, G) and the FGF target gene *chordin* (Fig. 1C), we postulate that the control of Pou5f1 on *fgf8a* and *chordin* expression is likely indirect and may involve an intermediate repressor of *fgf8a* expression.

Time-resolved expression profiles of the organizer genes and BMP pathway components in MZspg mutants

Analysis of in-situ hybridization data comparing wild-type and MZ*spg* expression levels (Reim and Brand, 2006) is often neither quantitative with respect to absolute levels of expression, nor precise with respect to developmental timing. Therefore, we utilized time-resolved microarray expression data (Onichtchouk et al., 2010), in which the vast abundance of probes enables normalization and comparison of the relative levels of transcripts between MZ*spg* and WT controls at 10 developmental time points from zygote to 8 hpf (Fig. 3A and C). First, we analyzed the temporal expression profiles of known BMP pathway antagonists (Fig. 3A). In agreement with our in-situ hybridization results (Fig. 2C and D), MZ*spg* embryos have precocious activation of *fgf8a* expression at sphere stage (4 hpf). Previous analysis implicated four FGF ligands in the regulation of *bmp* genes at blastula stage (Cao et al., 2004b; Furthauer et al., 2004). When analyzing expression of these FGF genes, we detected transient increases of expression in MZ*spg* at sphere also for *fgf17b* and *fgf3*, while *fgf24* expression was not detectable in both WT and MZ*spg* at this stage (Fig. S1A). In addition, expression of the BMP pathway

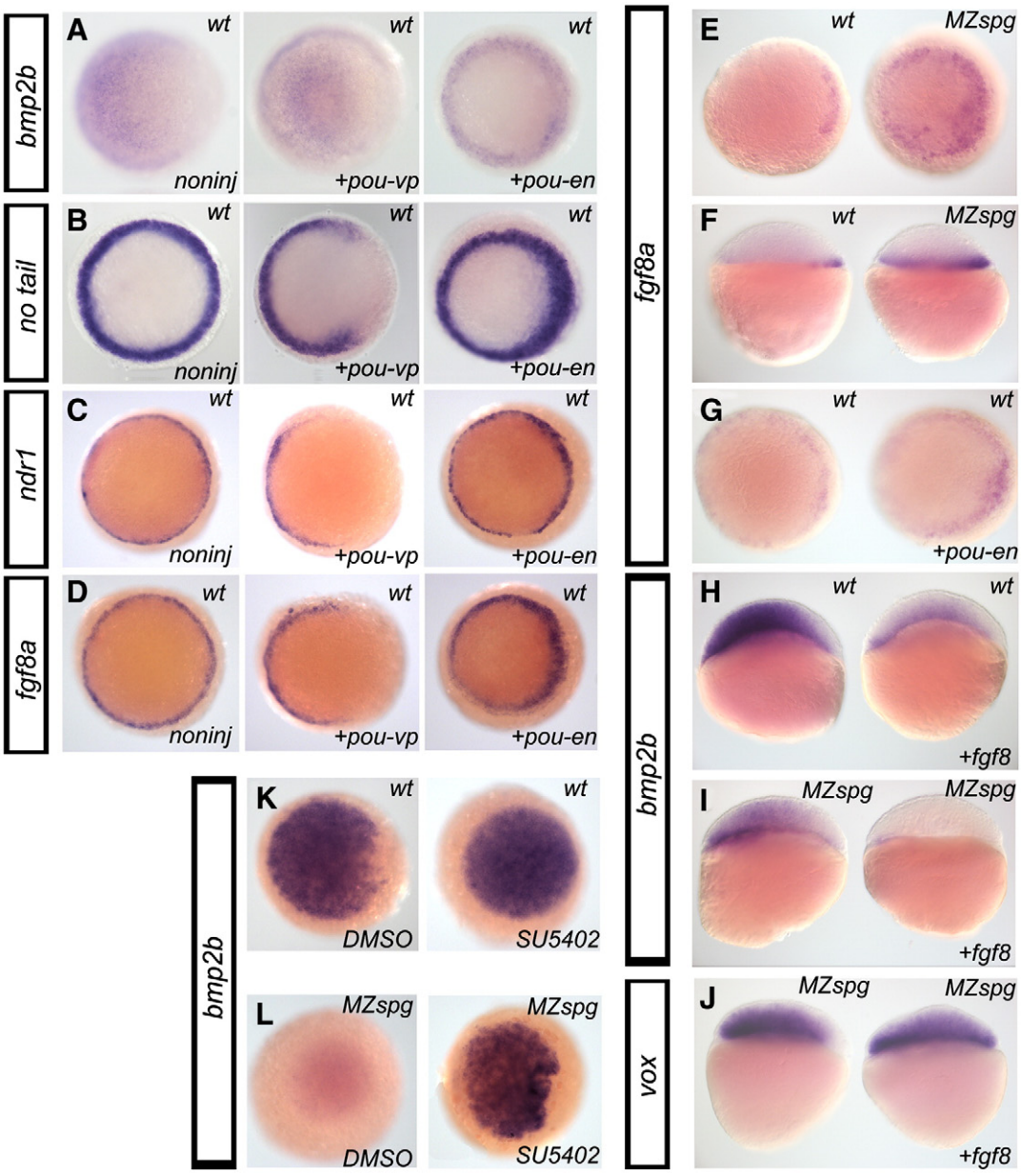

Fig. 2. Regulatory interactions of *pou5f1*, *fgf8a* and *bmp2b*. Whole-mount in situ hybridization of wild-type (WT) embryos and MZ*spg* mutants, injected with mRNAs as indicated. (A–D) WT embryos were left non-injected, or were injected with Pou5f1-VP16 (*pou-vp*) or Pou5f1-Engrailed (*pou-en*) encoding mRNAs, fixed at 40%–50% epiboly stage, and stained with the probes as indicated on the left sides of the panels. Animal views. Note the gap in the marginal ring of expression of *no tail*, *ndr1* and *fgf8a* for Pou5f1-VP16 overexpressing embryos, and local thickening of the marginal expression ring for Pou5f1-Engrailed overexpressing embryos (B–D). (E, F) Sphere stage WT embryos and MZ*spg* mutants probed with *fgf8a*. *fgf8a* is expanded in MZ*spg* embryos. (C) Animal view, (D) lateral view. (G) WT embryos were left non-injected (left), or were injected *pou5f1-En* mRNA (43 pg; right) and probed with *fgf8a*. *fgf8a* is expanded in *pou5f1-En* mRNA injected embryos (7, n = 8). Animal view, sphere stage. (H) WT embryos were left non-injected (left), or were injected with *fgf8a* mRNA (3 pg/embryo; right) and probed with *bmp2b*. *bmp2b* expression is reduced in 12 out of 14 *fgf8a* mRNA injected WT embryos analyzed. Lateral view, 30% epiboly. (I, J) FGF signaling is functional in blastula MZ*spg* embryos and can suppress *bmp2b* expression. MZ*spg* embryos were left non-injected (left), or were injected with *fgf8a* mRNA (3 pg/embryo) and probed with *bmp2b* (G) or *vox* (H). (G) *bmp2b* expression is further reduced in *fgf8a* mRNA injected MZ*spg* embryos (13, n = 14 embryos). (H) *vox* expression did not significantly change. Lateral views 30% epiboly. (K, L) Inhibition of FGF signaling restores *bmp2b* expression at sphere stage in MZ*spg* embryos. To inhibit FGF signaling, WT (K) and MZ*spg* (L) embryos were treated with 40 μM SU5402 (or controls with DMSO only) starting from 2-cell stage, fixed at sphere stage, and probed with *bmp2b*.

antagonists *noggin1* (Furthauer et al., 1999) and *chordin* show a similar behavior (Fig. 3A, arrows). *chordin* was shown to be a target of Nodal and FGF signaling (Dal-Pra et al., 2006; Maegawa et al., 2006; Varga et al., 2007), so it is possible that *chordin* activation occurs in response to upregulated FGF signaling. Levels of transcripts for the organizer genes *bozozok* and *ndr1 (squint)* (Fekany et al., 1999; Gritsman et al., 1999) at sphere stage are the same in WT and MZ*spg* (Fig. 3A, arrows). *admp* expression begins in the organizer at blastula stages, and its maintenance is positively influenced by Nodal and Boz (Lele et al., 2001). We measured relative expression of *admp* and *chd* by quantitative RT-PCR at several stages in MZ*spg* and WT embryos in parallel PCR reactions. While we confirmed a transient over-activation of *chd* expression at sphere stage, *admp* expression was significantly reduced in MZ*spg* (Fig. 3B). Thus, premature activation at sphere stage in MZ*spg* is not a property of all organizer genes.

Following the transient increase at sphere stage, *fgf8a* levels in WT and MZ*spg* were not significantly different until 8 hpf. *noggin 1* was increased in MZ*spg* after shield stage, while absolute levels of *chd* message were not increased in MZ*spg* mutants starting from shield stage (Fig. 3A and B). The temporal behavior of ventral genes in WT

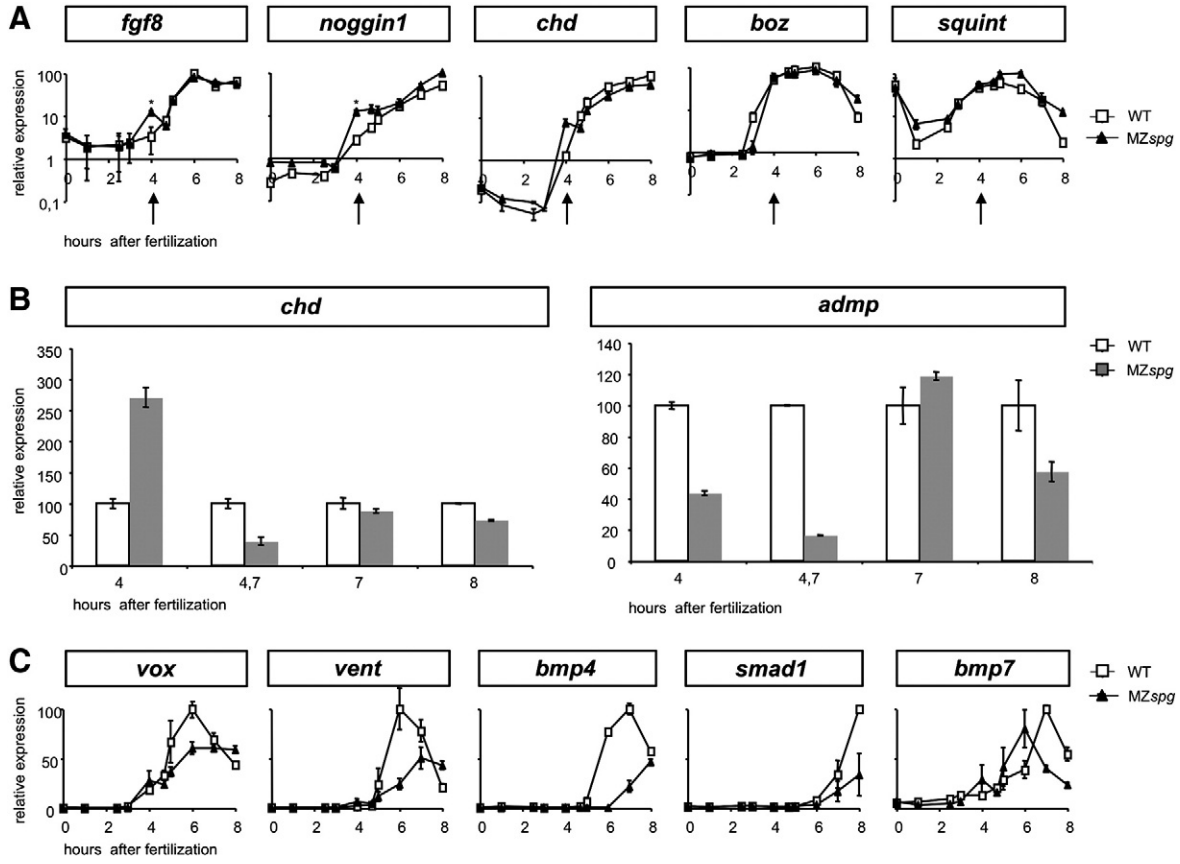

Fig. 3. Temporal expression profiles of BMP pathway genes in WT and MZ*spg*. (A, C) Microarray expression profiles for WT (white squares) and MZ*spg* (black triangles) embryos during the first eight hours of development. The analyzed genes are indicated above the graphs. The Y-axis shows relative expression values, which were normalized to the maximal expression value (100) measured for each gene. Error bars show SEM (standard error of the mean) of one (*fgf8*, *boz*, *squint*), two (*bmp4*, *noggin1*, *chordin*), or three (fgf8) microarray probe expression values, all averaged from three biological replicates. (A) BMP pathway antagonists *fgf8*, *noggin1* and *chd* are prematurely activated in MZ*spg* at sphere stage (indicated with arrow). Fold change and p-values in Student's T-test were 3.78×, $p = 0.012$ for *fgf8*, 4.57×, $p = 0.01$ for *noggin* 1 and 6.3×, $p = 0.057$ for *chd*. *Denotes statistically significant result ($p < 0.05$). *chd* premature activation which did not reach statistical significance, was confirmed by Real Time (RT)-PCR (B). (B) Temporal changes in *admp* and *chd* expression in WT and MZ*spg* assayed by quantitative RT-PCR. MZ*spg* expression values (gray bars) are shown relative to WT (white bars). The Y-axis shows normalized values, (WT is always 100%), and the X-axis shows embryonic stages: sphere (4 hpf), 30% epiboly (4.7 hpf), 60% epiboly (7 hpf) and 75% epiboly (8 hpf). Error bars denote SEM from three technical replicates. (C) Temporal expression profiles of selected BMP pathway components showing significant expression level changes between WT and MZ*spg*. For organization of graphs, see legend for (A).

and MZ*spg* is shown in Fig. 3C. While *vox* and *vent* expression initiated at the same time in WT and MZ*spg* (4 hpf and 5 hpf, respectively), levels of both dramatically differ at shield stage (6 hpf), but become more WT-like later during gastrulation in MZ*spg*. Zygotic activation of *bmp4* expression was delayed from 30% epiboly to shield stage (6 hpf). Expression levels of the signal transducer *smad1* were significantly reduced during late gastrulation (8 hpf, 75% epiboly), which is compatible with its regulation by BMP signaling (Dick et al., 1999). *bmp7* expression levels in MZ*spg* became reduced in comparison to WT during gastrulation.

BMP signaling is active but reduced in MZspg mutants

In agreement with previous results (Reim and Brand, 2006), during mid-to-late gastrulation of MZ*spg* mutants, *bmp2b*, *vox* and *vent* expression are decreased and shifted ventrally, and the *chordin* domain expanded ventrally (Fig. 4A and B). Failure of normal *bmp4* initiation on the ventral side (Figs. 3C, 4A) and severe reduction of the ectodermal transcription factors *gata2*, *tfap2a*, *foxi*, *dlx3b* and *tp63* (Fig. S1B) at late gastrulation stages in MZ*spg* introduce the possibility that BMP signaling is blocked in these embryos. We injected a constitutively active form of BMP-receptor 1a (*caBMPR1a*, *caAlk3a*) mRNA (Nikaido et al., 1999) at the one-cell stage to determine whether MZ*spg* embryos can be ventralized by activation of BMP signaling. Injected embryos developed until late gastrulation, and were then assayed for expression of *chd* and *vox*. We found that injection of *caBMPR1a* mRNA can expand marginal expression of *vox* toward the dorsal side, and restrict the lateral domains of *chd* dorsalization in *MZspg* mutant embryos (Fig. 4B) starting only after 70% epiboly, but not before (data not shown). Thus, judged by *vox* expression, MZ*spg* embryos are able to respond to BMP signaling within the same time window as WT.

Zygotic Smad1/5 signaling is severely affected in *bmp7* and *bmp2b* null mutants (Mullins et al., 1996; Tucker et al., 2008). Nuclear localization of P-Smad is the most direct readout for active BMP signaling. To analyze changes in zygotic Smad1/5 signaling activity in MZ*spg* embryos, we stained MZ*spg* and WT control embryos with anti-Phospho-Smad1/5 antibody, which reveals the active phosphorylated form of Smads 1, 5 and 8. The dorsoventral gradient of active BMP signaling is clearly visible in MZ*spg* (Fig. 4C), although P-Smad1/5 staining is shifted ventrally both in the mesoderm and the ectoderm. This reveals that Bmp2/Bmp7 heterodimers are present in MZ*spg* embryos, although at lower concentrations than in WT controls.

vox is a direct transcriptional target of Pou5f1 following MBT

Since the most dramatic differences in the expression patterns and transcript abundance of BMP pathway components were seen at early stages, we asked whether there is a direct zygotic activation of BMP

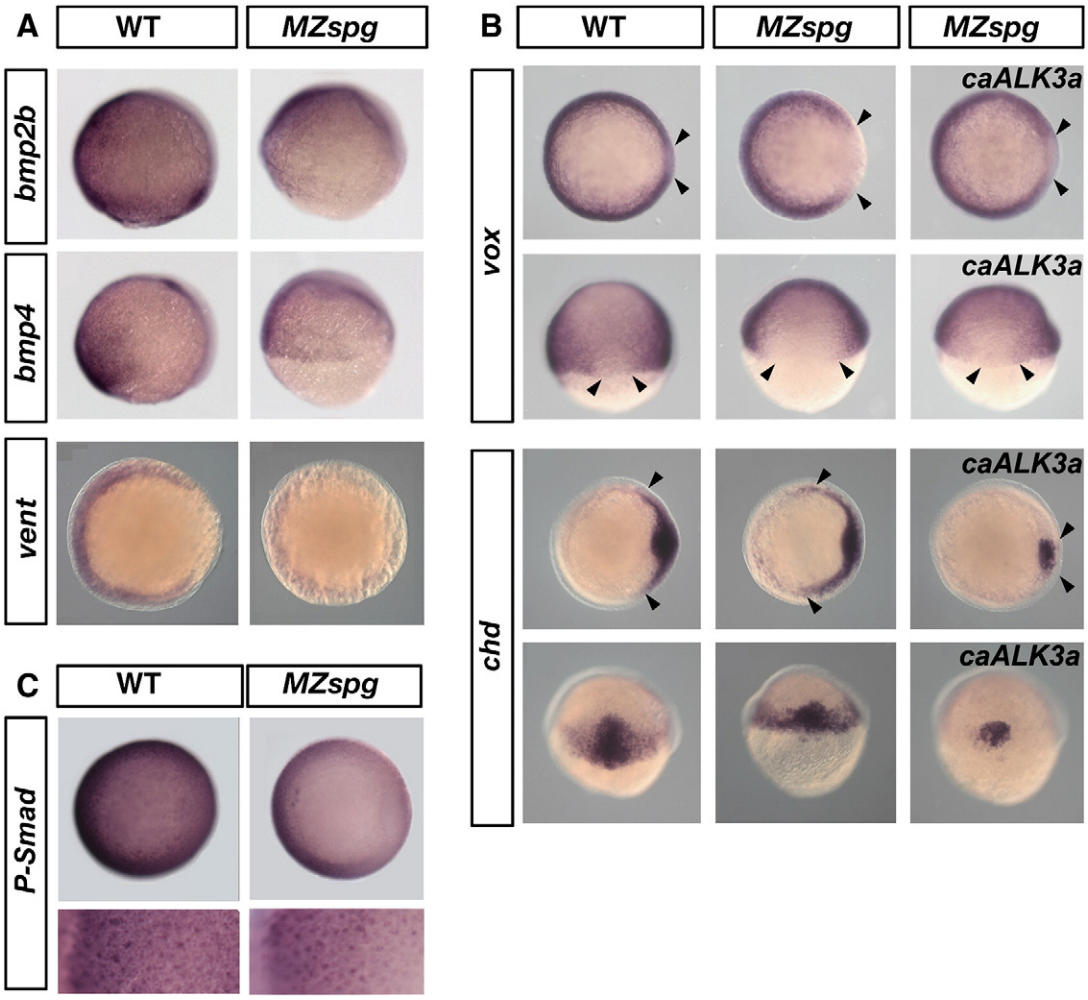

Fig. 4. BMP signaling and expression of zygotic BMP pathway genes in MZ*spg*. (A) Expression of BMP pathway genes is reduced in MZ*spg* embryos. From top to bottom: Whole-mount in-situ hybridization of wild-type (WT) embryos and MZ*spg* mutants showing expression of *bmp2b*, *bmp4* (lateral view, dorsal to the right, 75% epiboly) and *vent* (animal view, dorsal to the right, shield stage). (B) Constitutively active BMP receptor 1a (caALK3a) can rescue dorsalization of MZ*spg* embryos at midgastrulation. Whole-mount in situ hybridization of wild-type (WT) embryos, MZ*spg* mutants, and MZ*spg* mutants injected with *caALK3a* mRNA, as indicated. From top to bottom: *vox* at 70% epiboly, *chordin* at shield stage; top rows: animal pole views, dorsal at right; bottom row: dorsal view, animal pole up. Arrows show the dorsolateral borders of *vox* and *chd* staining. *caBMPR1a* mRNA (100–150 pg) was injected into wild-type (WT) embryos at the one-cell stage. (C) Immunostaining for Phospho-Smad 1/5/8 (animal view, dorsal at right) of WT and MZ*spg* embryos at 75% epiboly. Lower panel shows animal-ventral view of the embryos in higher magnification, to visualize P-Smad nuclear staining from ventral margin (left) toward the animal pole (right).

pathway genes by Pou5f1 during gastrulation. Although previous studies (Reim and Brand, 2006) and our results suggest that Pou5f1 maintains *vent* and *vox* transcription during gastrula stages, it is unclear whether this is a direct (through Pou5f1-induced activation) or indirect (through intermediate transcriptional regulator) effect. We injected Pou5f1-VP16 mRNA into one-cell stage WT embryos and assayed injected and non-injected embryos at 30% epiboly by in-situ hybridization for *vent*, *vox*, *bmp2b*, *bmp4* and *no tail* (Fig. 5A and data not shown). We performed parallel experiments with or without addition of the translation inhibitor cycloheximide (CHX) from the 16-cell stage onwards to suppress indirect gene activation or inhibition from translation of zygotic mRNAs after MBT (Leung et al., 2003). *no tail* induction requires intermediate zygotic translation steps after MBT; thus the absence of *no tail* signal served as a control for efficiency of CHX treatment. In the presence of CHX, *vox* (47 embryos, n = 53) and *vent* (37 embryos n = 40) expression was robustly upregulated by Pou5f1-VP16 in WT embryos, but *bmp2b* levels were indistinguishable from controls (Fig. 5A). *bmp4* expression was not induced (data not shown), indicating that its expression is controlled by zygotic factors and not solely controlled by Pou5f1. In MZ*spg* embryos in the presence of CHX, Pou5f1-VP16 over-expression induced strong expression of *vox* but not *vent* (Fig. 5B). We confirmed by quantitative RT-PCR that overexpression of Pou5f1-VP16 or Pou5f1 activated, while Pou5f1-En repressor construct repressed *vox* transcription in the presence of CHX. (Fig. 5C and D). We conclude that *vox* is a direct transcriptional target of Pou5f1.

Pou5f1 activates the vox promoter using phylogenetically conserved Oct4 binding site

We searched the upstream genomic region of *vox* for Pou5f1 and octamer binding sites, and found four well-matching sequences within 1 kb immediately upstream of the TATA-box. We cloned this 1 kb *vox* promoter fragment into a luciferase reporter construct (Vox-luc) and performed luciferase assays for WT and MZ*spg* embryos at shield stage. Normalized luciferase activity of the Vox-luc reporter in WT was an order of magnitude higher than in MZ*spg* embryos (Fig. 6A). Pou5f1 overexpression in MZ*spg* embryos resulted in up to four-fold activation of the luciferase activity. The Vox-luc reporter also responded with two-fold activation to *smad1* mRNA overexpression; co-injection of *pou5f1* and *smad1* mRNAs activated Vox-luc in an additive fashion (Fig. 6B).

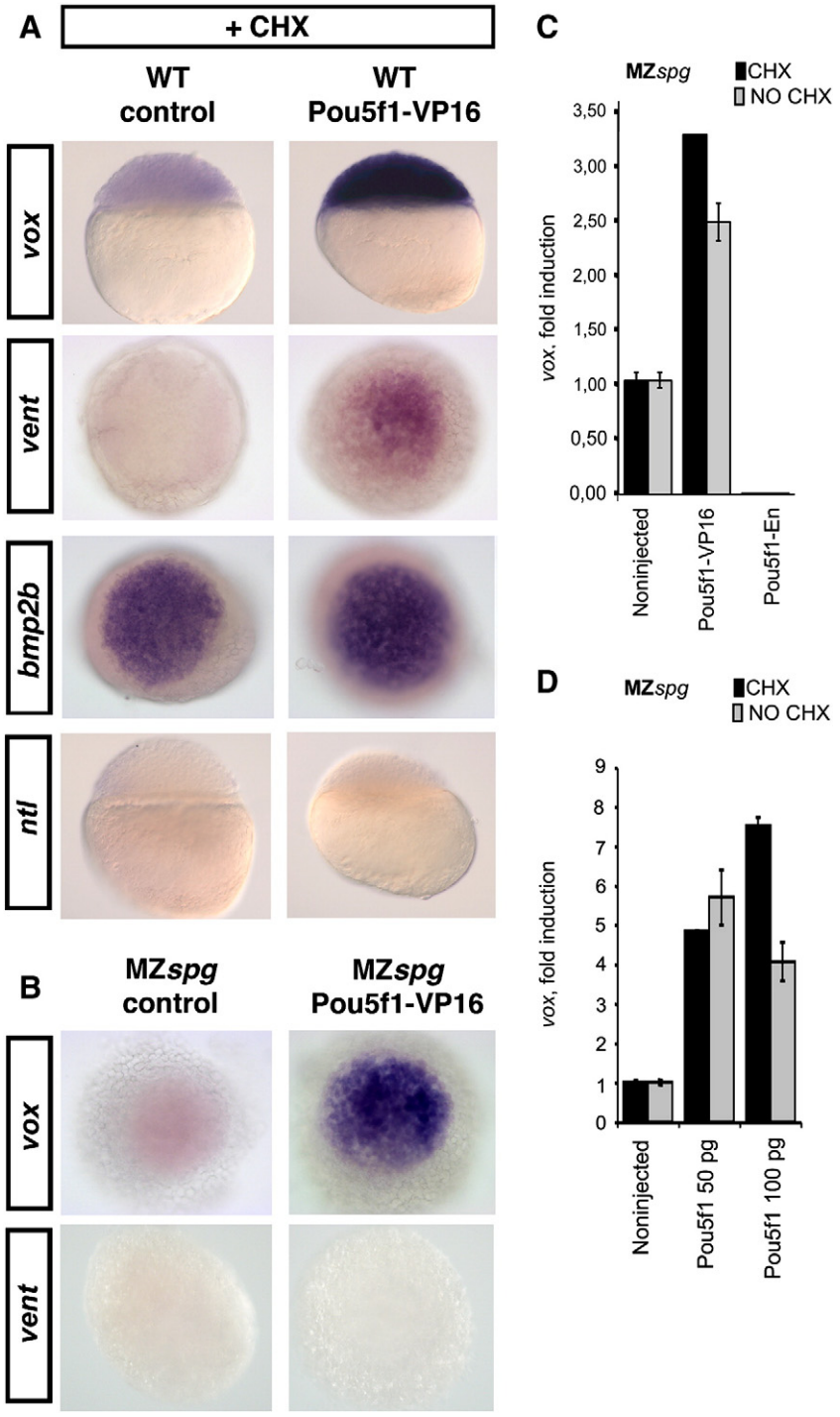

Fig. 5. *vox* is a direct transcriptional target of Pou5f1. (A) Whole-mount in situ hybridization of 5 hpf wild-type control embryos and embryos injected with Pou5f1-VP16; probes are as indicated. Embryos were treated with cycloheximide (CHX) starting at the 64-cell stage to prevent translation. Absence of *ntl* staining served as a control for zygotic translation block. Note that Pou5f1-VP16 induced *vent* and *vox* genes. Embryos probed with *ntl* and *vox* are shown in lateral view, and embryos probed with *bmp2b* and *vent* stained embryos are shown in animal view. (B) Whole-mount in situ hybridization of CHX-treated non-injected MZ*spg* embryos and MZ*spg* embryos injected with *pou5f1-VP16* and analyzed with *vox* or *vent* probes, animal view (44 out of 48 embryos showed increased *vent* expression). (C, D) Quantitative PCR: *vox* mRNA levels were measured in CHX treated and untreated shield stage MZ*spg* embryos, which were non-injected, or injected with mRNAs at one cell stage as indicated. *vox* expression values were normalized to housekeeping gene *ef1alpha* expression levels (ddCT method) and then to non-injected control (set as 1). (C) Pou5f1-VP16 induces *vox*, and Pou5f1-En repressed *vox* expression in the presence of CHX in MZ*spg*. (D) Pou5f1 induces *vox* in the presence of CHX in MZ*spg*.

The upstream region of *vox* included in the Vox-luc plasmid (Zv7: chr13: 52,347,159–52,348,135) contains four putative Oct4/Pou5f1-binding sites, which we numbered P1 to P4. P1 and P2 lie within the region 200 bp upstream of the *vox* TATA-box, which is phylogenetically conserved among five fish species (Fig. 7A) and includes a conserved BMP response element (BRE) (Yao et al., 2006). We performed gel-retardation assays using labeled P1–P4 oligos and found that both zebrafish Pou5f1 and mouse Oct4 protein were able to bind to P1 specifically (Fig. 7B, Fig. S2). To determine whether these in-vitro results are relevant in the embryo, we performed chromatin immunoprecipitation (CHIP) assays with extracts from shield stage embryos injected with mRNA encoding FLAG-tagged Pou5f1. In the

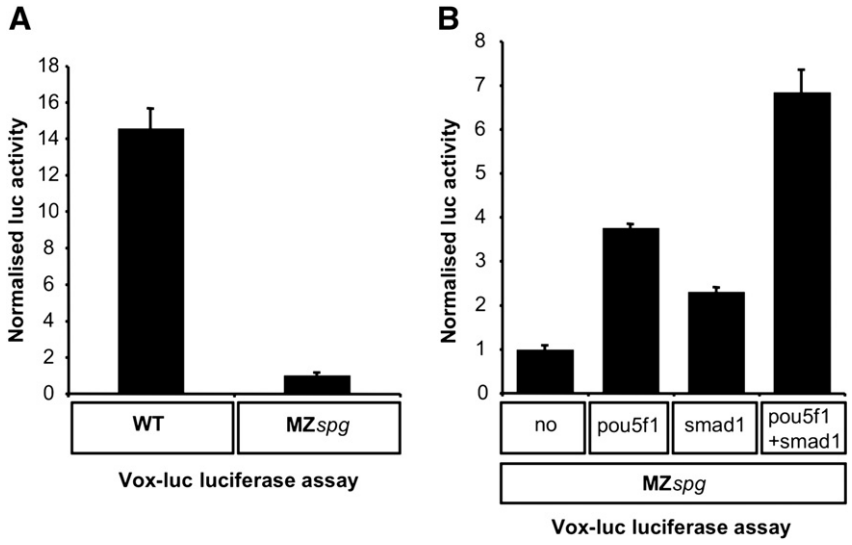

Fig. 6. A 1 kb *vox* promoter fragment mediates Pou5f1-dependent expression of *vox*. (A, B) Luciferase assays: activity of Vox-luc reporter plasmid was measured in WT and MZ*spg* (A), and in MZ*spg* embryos injected with *pou5f1* and/or *smad1* expression constructs, as indicated (B).

immunoprecipitate from the injected sample, we saw a significant enrichment for the region immediately upstream of the *vox* transcription start site (Fig. 7A) when compared to the noninjected control. The control genomic region (*rpl5b*) did not show enrichment (Fig. 7C). These data demonstrate that *vox* is a direct zygotic target of zebrafish Pou5f1.

Discussion

Mechanistic links between the control of pluripotency and embryonic pattern formation in early blastula and gastrula stages are not well understood, despite the fact that specific signaling pathways contribute to both aspects of development. One example is BMP signaling, which contributes to sustaining embryonic stem cell self-renewal in mouse (Ying et al., 2008) as well as dorsoventral pattern formation (De Robertis et al., 2000) and many induction processes in development. One could hypothesize that there may be mechanistic links between temporal release from an early non-determined cell state and the progress of dorsoventral patterning. Evidence that Pou5f1 contributes to dorsoventral patterning comes from *pou5f1* mutant MZ*spg* zebrafish (Reim and Brand, 2006), as well as from activities of the Pou5f1 homologue Oct-25 in *Xenopus* (Cao et al., 2004a; Morrison and Brickman, 2006).

Here we made use of activating (Pou5f1-VP16) and repressing (Pou5f1-En) constructs to further investigate direct and indirect effects of Pou5f1 on expression of genes implicated in dorsoventral patterning. We show that Pou5f1 contributes to BMP pathway regulation via at least two mechanisms: direct transcriptional activation of *vox*, and indirect control of *fgf8a* and organizer gene expression shortly after MBT. Absence of Pou5f1 in the embryo reduces, but does not completely abolish BMP signaling at the end of gastrulation.

Pou5f1 contributes to activation of developmental genes

To address whether Pou5f1 functions as an activator or as a repressor, we used activating (Pou5f1-VP16) and repressing (Pou5f1-En) fusion proteins. Low doses of Pou5f1-VP16 can rescue the gastrulation defects, endoderm formation, and epiboly defects in MZ*spg* mutant. Pou5f1-En had no rescue effect (Lunde et al., 2004), suggesting that transcriptional activation is the predominant mode of Pou5f1 action in zebrafish development.

However, in contrast to Pou5f1-VP16 and Pou5f1-En fusions, overexpression of unconjugated Pou5f1 fails to ventralize WT embryos (Reim and Brand, 2006; Takeda et al., 1994), and also fails to perturb the expression of *ntl*, *fgf8a* or *ndr1* in WT embryos (Fig. 2B–D, and data not shown). These data are compatible with a model in which Pou5f1 upon binding to target gene promoters acts predominantly to permit transcriptional activation of those genes in concert with other activating factors, rather than providing most of the activating function itself. Pou5f1 may thus activate targets only in a dynamic and tissue-specific association with other transactivating factors. A similar mode of action of Pou5f1 has been suggested previously also for mouse Pou5f1/Oct4 (Niwa et al., 2002).

Regulation of fgf8a and bmp2b shortly after MBT

Blastula and early gastrula *bmp2b* expression, normally initiated at sphere stage, is absent in MZ*spg* embryos, and a direct input of maternal Pou5f1 to the activation of *bmp2b* has been suggested (Reim and Brand, 2006). However, in our experiments, overexpression of Pou5f1-VP16 did not affect *bmp2b* expression at blastula stages, suggesting that additional factors may be involved. Pou5f1-En overexpression downregulated *bmp2b* in the animal region, but not in the margin. A similar pattern of transcriptional downregulation of *bmp2b* has previously been observed in zebrafish following overexpression of Fgf8a or Fgf3 (Furthauer et al., 2004), suggesting that the Pou5f1-En overexpression effect on *bmp2b* may be indirect and may be mediated by activation of FGF signaling. We demonstrated that *fgf8a* transcripts, expressed dorsally in WT, extend over the marginal zone by sphere stage in MZ*spg* mutants (Fig. 2C and D), and that the amount of *fgf8a* transcripts in MZ*spg* embryos is transiently increased at sphere stage when compared to WT (Fig. 3A). We demonstrated that inhibition of FGF signaling restores initiation of *bmp2b* expression in MZ*spg* at sphere stage (Fig. 2K, L), similar to results obtained previously using the XFD dominant negative FGF receptor (Reim and Brand, 2006). These findings make transcriptional repression mediated by targets of FGF a plausible explanation for delayed initiation of *bmp2b* in MZ*spg* embryos. In addition, Pou5f1-VP16 downregulated and Pou5f1-En expanded the expression of *ndr1*, *fgf8a*, and of *no tail*. *no tail* is a target of both Nodal and FGF pathways (Griffin et al., 1995; Gritsman et al., 1999), therefore the effect of Pou5f1 on *ntl* may be explained by modulation of expression of both signals. We interpreted our data as indirect interference of Pou5f1 with *fgf8a* expression via an unknown transcriptional repressor (i.e.

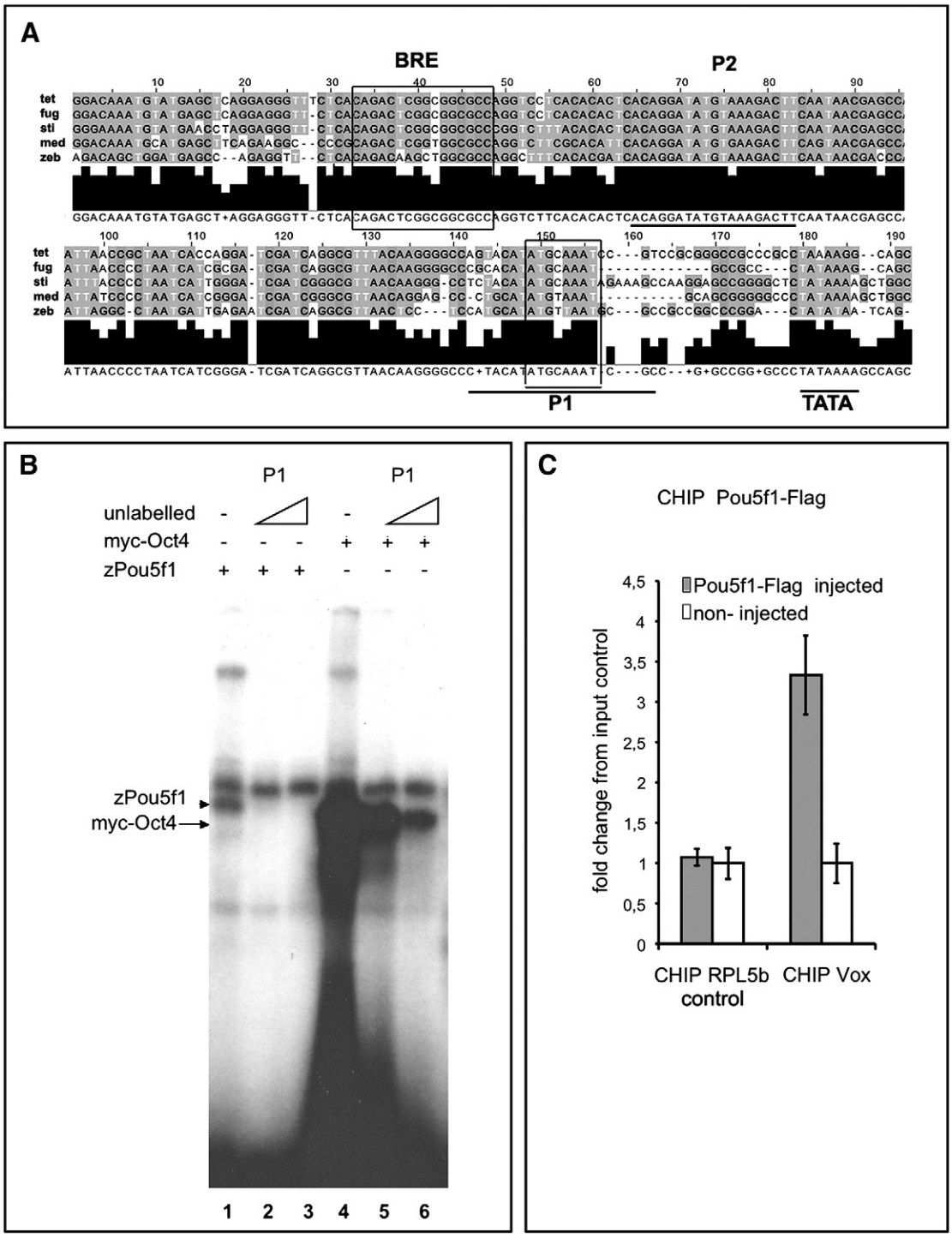

Fig. 7. The *vox* promoter contains phylogenetically conserved sites that bind Pou5f1 in vitro and in vivo. (A) Alignment of *vox* upstream regions of five teleost fish genomes. Tet: Tetraodon; fug: Fugu; sti: stickleback; med: medaka; zeb: zebrafish. Underlined regions: TATA — TATA box, P1, P2-oligos used for gel shift. Boxed regions: P1 — Pou5f1 binding site 1; BRE — conserved BMP-responsive element (Karaulanov et al., 2004; Yao et al., 2006) (see Materials and methods for the sequence annotations). (B) Gel retardation assay, with labeled P1 DNA probe; zebrafish Pou5f1 protein (zPou5f1, lanes 1–3) or myc-tagged Oct4 protein (lanes 4–6) was used. Reactions were performed with or without excess of unlabelled P1 oligo (lanes 1 and 4 — no unlabelled, lanes 2 and 5 — 10 fold excess; lanes 3 and 6 — 50 fold excess). zPou5f1 forms a complex with P1 oligo (arrowhead, lane 1); complex formation can be specifically inhibited by excess of unlabelled P1 oligo (lanes 2, 3). Mouse myc-Oct4 forms a complex with P1 oligo (lane 4, arrow); complex formation can be specifically inhibited by excess of unlabelled P1 oligo (lanes 5, 7). (C) CHIP (chromatin immunoprecipitation) of Pou5f1-FLAG injected embryos with anti-FLAG antibody. Chromatin from Pou5f1-FLAG injected (gray bars) and non-injected (white bars) embryos was sonicated and immunoprecipitated with anti-FLAG antibody. DNA levels of Vox upstream region and control sequence (RPL5b exon) were measured by QPCR and normalized to input control levels. Upstream *vox* region (Zv7: chr13: 52,347,978–52,348,087) is 3× enriched in the immunoprecipitated sample in comparison with the input control ($p = 0.0364$ in Student's T-test). Non-injected control shows no enrichment (CHIP Vox). RPL5b control shows no enrichment in injected or non-injected sample (CHIP RPL5b). Error bars: SEM (standard error of the mean).

Pou5f1 activates ventral transcriptional repressor X, which inhibits *fgf8a* expression, Fig. 8A). Vox is expressed early and has been shown to have repressor activity (Kawahara et al., 2000a), which together makes Vox a likely candidate for repressor X function. However, even in combination with Vent, Vox is not sufficient to account for repressor X activity, because the expression of *bmp2b* at blastula

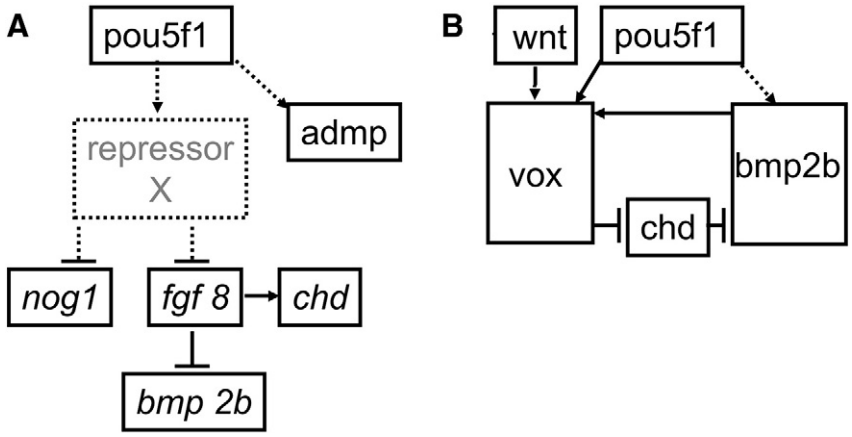

Fig. 8. Schematic drawing of Pou5f1 interactions with dorsoventral patterning. Dotted arrows show direct or indirect activation or repression, and solid arrows show direct activation or repression. Only the interactions presented in the Results section are shown. (A) MBT-early blastula stage. At MBT maternal Pou5f1 is postulated to activate a short-living repressor X, or a group of redundant repressors, which repress *fgf8*, *noggin* and *chd*. *bmp2b* is transcriptionally repressed by FGF signaling (Noggin and Chordin do not repress BMP2b expression at blastula stage (Furthauer et al., 2004)). Pou5f1 activates *admp* expression by unknown mechanisms. (B) Late blastula and gastrula stages. Pou5f1 participates in direct activation of *vox*, repressor of dorsal genes (*chordin*). Regulation of *bmp2b* by Pou5f1 is indirect. *vox* expression depends on BMP signaling from midgastrula stages onwards, thus the *pou5f1* mutation in MZ*spg* may be rescued by an increase in BMP signaling.

stages is normal even in the embryos lacking both Vox and Vent functions (Imai et al., 2001). It is thus possible that Vox may contribute to repressor X activity, but if so, it may act redundantly with other factors.

Functions of early pathways patterning the embryo should be precisely tuned, so that desired signaling levels are achieved at the desired time. Taken together, our data suggest that one of the functions of Pou5f1 is to ensure, via indirect mechanisms, proper temporal control over activation of FGF signaling and dorsal organizer genes at, or shortly after, MBT. The differential regulation of organizer molecules in MZ*spg* mutants suggests that Pou5f1 controls the temporal and spatial synchronization of organizer function downstream of Nodal and Wnt pathways.

Transcriptional activation of vox and vent genes by Pou5f1

MZ*spg* embryos have physically enlarged organizers, similar to *vox* and *vent* mutants, but in contrast to zebrafish BMP pathway mutants *swirl* and *somitabun* (Imai et al., 2001; Kawahara et al., 2000a, 2000b; Mullins et al., 1996; Miller-Bertoglio et al., 1997; Ramel and Lekven, 2004). This defect can be rescued by injection of activated forms of BMP receptors Alk8 (Reim and Brand, 2006) or Alk3 (Fig. 4B), which activate *vox* and *vent* genes during late gastrulation.

In contrast to *bmp2b*, following Pou5f1-VP16 expression in WT and CHX treatment, *vox* appears to be a direct target of Pou5f1, while *vent* is upregulated to a lesser extent. However, *vent* was not induced by Pou5f1-VP16 in MZ*spg* embryos in the presence of CHX, indicating that some components necessary for *vent* activation are missing in MZ*spg* embryos. *vent* induction could fail in MZ*spg* embryos due to insufficient levels of BMP signaling or reduced Vox levels. *vent* has been shown to be more dependent on BMP signaling than *vox* (Melby et al., 2000). Further, *Xenopus Xvent-1*, which is a functional homologue of zebrafish *vent*, also requires higher BMP signaling level to be expressed (Onichtchouk et al., 1998). In *Xenopus*, the Xvent-1B gene is activated by Xvent-2B/Vox (Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996) in cooperation with Gata2, and BMP signaling Smads (Friedle and Knochel, 2002; Rastegar et al., 1999). Although there is no evidence that Vox in zebrafish is also required for *vent* expression, the Pou5f1 direct regulatory input seems to be stronger for *vox* than for *vent*. Pou5f1 binds within 200 bp of the *vox* promoter upstream of the TATA-box, in close proximity to a BMP response element (BRE), that is repeatedly found in regulatory regions of BMP responsive genes in vertebrates (Karaulanov et al., 2004), and is thought to be the vertebrate correlate of the *Drosophila* Dpp response element (Yao et al., 2006). In-vivo CHIP assay confirms Pou5f1 binding to the *vox* proximal upstream region. Smad1 and Pou5f1 can activate a Vox-luc reporter in an additive manner (Fig. 6B). A similar arrangement of Smad and POU binding sites has been described for the *Xvent-2B* promoter, which is activated by Xlpou25 (Cao and Zhang, 2004). A summary of Pou5f1 interactions with selected dorsoventral patterning genes during gastrulation is shown in Fig. 8B.

Other interactions between the BMP pathway and the Pou5f1 downstream transcriptional network

Robustness of the BMP dorsoventral patterning system and its ability to self-regulate is based on multiple partially redundant pathway feedback loops, working in parallel to buffer against fluctuations in gene dosage and expression (Ben-Zvi et al., 2008; Eldar et al., 2002). This feature makes epistatic analysis particularly challenging, because perturbations of earlier events can be compensated by self-regulating systems during later development. Zebrafish Pou5f1 directly activates multiple targets in all germ layers (Onichtchouk et al., 2010), suggesting that interactions of the Pou5f1 transcriptional network with BMP pathway targets occur at multiple levels of regulation. Absence of Pou5f1 does not block the BMP pathway, as staining for active nuclear localization of P-Smad1/5/8 shows that during midgastrulation BMP pathway signaling is active on the ventral side of MZ*spg* embryos. P-Smad1/5/8 forms a gradient both in the marginal zone and the ectoderm, although the borders of active Smad are shifted ventrally in MZ*spg* (Fig. 4C). The relatively mild effect on BMP signal transduction in MZ*spg* embryos is not sufficient to explain the failure to initiate *bmp4* expression (Fig. 3C) and complete loss of epidermal markers *foxi* (Dee et al., 2007), *gata2*, *tfap2a* (Nguyen et al., 1998), *dlx3b* (Akimenko et al., 1994) and *tp63* (Lee and Kimelman, 2002) seen in MZ*spg* embryo (Fig. S1B; also Reim and Brand, 2006). Recent transcriptome studies (Okuda et al., 2010; Onichtchouk et al., 2010) suggest that SoxB1 transcriptional activators, in addition to regulation by BMP signaling, regulate *bmp4*, *foxi*, *gata2*, *tfap2a*, and *dlx3b*. There are four SoxB1 group genes active during the blastula–gastrula stages in zebrafish, Sox2, Sox3, Sox19a, and Sox19b, and all four share redundant functions (Okuda et al., 2010). We have shown in our recent paper (Onichtchouk et al., 2010) that *sox2* and *sox19b* transcription are severely downregulated in MZ*spg* embryos. As transcriptional activity of Sox2 and Sox19b is severely reduced in MZ*spg* embryos (Onichtchouk et al., 2010), strong

downregulation of *bmp4*, *foxi*, *gata2*, *tfap2a* and *dlx3b* transcripts is most likely due to a combined reduction of Smad1/5 signaling and SoxB1 class transcriptional activation in MZ*spg* mutant embryos.

Pou5f1 and BMP pathway interactions

Dramatic loss of *bmp4* and *xvent-2* (functional homologue of *vox*) expression was observed in *Xenopus* in the triple knockout for Xlpou60, Xlpou25 and Xlpou91 (Morrison and Brickman, 2006), arguing for conserved interactions between the Pou5f1 and BMP pathways in *Xenopus* and zebrafish. Interestingly, at least two of the Oct4 target lists derived from microarray and CHIP studies in mouse ES cells contain *bmp4* (Loh et al., 2006; Sharov et al., 2008), suggesting that BMP pathway regulation by Pou5f1 is phylogenetically conserved.

Our data and recently published work (Onichtchouk et al., 2010) reveal that Pou5f1 may contribute not only to maintaining the undifferentiated state, as judged from control of expression of Sox genes and of repressors of differentiation, but may also contribute in an intricate fashion to proper integration of developmental timing and patterning mechanisms. Levels of BMP pathway activation and duration of BMP signal are both critical for embryonic patterning: in particular, distinct temporal intervals of BMP signaling are required for correct coupling of dorsoventral and anterioposterior cell fates (Tucker et al., 2008). In MZ*spg* embryos, the strength of BMP signaling is lower, as shown by P-Smad staining, and the duration of BMP signaling activity is shorter, due to the inhibition by FGF pathway shortly after mid-blastula transition. Thus, Pou5f1 is involved in fine-tuning of both the activity levels and signal duration of the BMP network. While the exact roles of Pou5f1 in the early signaling and patterning networks still need to be elucidated, we hypothesize that Pou5f1 may act as a temporal cue to regulate both the temporal dynamics of BMP levels in a cell and the competence to respond in executing a position specific differentiation program. These mechanisms may contribute to the sophisticated signaling networks that enable gastrula cells to acquire dorsoventral and anterioposterior identities simultaneously, and with respect to the whole embryo, in a defined temporal order.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.05.660.

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